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Investigating cellular stress response to heat stress in the seagrass *Posidonia oceanica* in a global change scenario

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ABSTRACT

Posidonia oceanica meadows are facing global threats mainly due to episodic heat waves. In a mesocosm experiment, we aimed at disentangling the molecular response of *P. oceanica* under increasing temperature (20 °C–32 °C). The experiment was carried out in spring, when heat waves can potentially occur and plants are putatively more sensitive to heat stress, since they are deprived in carbohydrates reserves after the cold winter months. We aimed to identify the activation of different phases of the cellular stress response (CSR) reaction and the responsive genes activated or repressed in heated plants. A molecular traffic light was proposed as a response model including green (protein folding and membrane protection), yellow (ubiquitination and proteolysis) and red (DNA repair and apoptosis) categories. Additionally, we estimated phenological trait variations to complement the information obtained from the molecular proxies of stress. Despite reduced leaf growth rate, heated plants did not exhibit signs of irreversible damage, probably underlying species pre-adaptation to warm and fluctuating regimes. Gene expression analyses revealed that molecular chaperoning, DNA repair and apoptosis inhibition processes related genes were the ones that mostly responded to high thermal stress and will be target of further investigation and *in situ* proofing for assessing their use as indicators of *P. oceanica* performance under sub-lethal heat stress.

1. Introduction

Seagrasses are keystone primary producers, “ecosystem engineers” responsible for the biophysical/chemical footprint of the coastal environment (Jones et al., 1994). They host a complex network of interacting organisms, providing them with trophic resources, nursery grounds, shelter and living substrate (Duarte, 2000). Covering nearly 0.2% of the global ocean, seagrass meadows represent ecological units with high biodiversity, which supply important ecosystem services, thus deserving conservation efforts as biological and economic value (Costanza et al., 1997; Heip et al, 1998; Duarte, 2000, 2002). These extensive marine prairies take an active role in climate regulation, biogeochemical cycling, local nutrient dynamics as well as sequestration of organic matter pools into millennial carbon-rich sediments; they also contribute to seafloor stability, preventing erosion and sediment resuspension (Mateo et al., 1997; Duarte, 2000; Marbà et al., 2006; Bos et al., 2007; Macreadie et al., 2014a). Furthermore, extending from shallow waters down to more than 50 m depth, seagrass beds overwhelmingly influence local hydrodynamics, attenuating wave energy and currents, ensuring coastline stability and water clarity (Gambi et al., 1990; Duarte, 1991; Pasqualini et al., 1998). *Posidonia oceanica* meadows represent the Mediterranean hotspots of benthic primary production and biodiversity, playing pivotal roles in ecosystem functioning (Procaccini et al., 2003). *P. oceanica* is included in the Red List of marine threatened species of the Mediterranean and meadows are defined as priority natural habitats on Annex I of the EC Directive 92/43/EEC on the Conservation of Natural Habitats and of Wild Fauna and Flora.

Since the early 20th century, seagrasses have been experiencing a global crisis, as highlighted by several reports of mortality events,

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decreased coverage and associated biodiversity loss worldwide (Orth et al., 2006 and e.g. Telesca et al., 2015; Thomson et al., 2015). Loss of meadows has been attributed to the combined effects of direct human activities (i.e. habitat fragmentation, eutrophication, pollution, overfishing, and biological invasions) and global climate change, both challenging their adaptability (Waycott et al., 2009; Doney et al., 2012). A broader awareness about these threats resulted in the concern that ecosystem benefits provided by seagrass meadows would be lost within a few decades (Duarte, 1999, 2002). Among the most hazardous signatures of global climate change, ocean warming is considered the most severe (Repolho et al., 2017; Duarte et al., 2018) and heat waves are responsible for dramatic seagrass deterioration, affecting photophysiological performances and thus survival (Winters et al., 2011; Moore et al., 2014; Thomson et al., 2015). These periods of abnormally high temperatures occur from late spring/early summer to early autumn (Rebetez et al., 2009) and are often associated with mortality events of marine flora and fauna, as reported in 2003 and 2006 (Coma et al., 2009; Hoegh-Guldberg and Bruno, 2010). The timing of anomalous heat events strongly affects the severity of their effects, as observed in trees, grass and other terrestrial plants (Teskey et al., 2015; Wang et al., 2016; De Boeck et al., 2011). The increase in average annual maximum surface sea-water temperature (SST; 26.6 °C–28.6 °C; http://www.puertos.es/es-es/oceanografia/Paginas/portus.aspx) following heat waves in the Western Mediterranean led to significant *in situ* shoot mortality and overall decline in *P. oceanica* across the Balearic Islands (Diaz-Almela et al., 2009; Marbà and Duarte, 2010). In the light of these findings, Jordà et al. (2012) predicted future trajectories of *P. oceanica* meadows in the Balearic Archipelago in an ocean warming scenario, excluding cumulative anthropic impacts and assuming a maximum mean annual SST of 28 °C. According to IPCC projections, heat waves are expected to increase in frequency and intensity and the Mediterranean SST is predicted to rise by 3.5 °C (Meehl and Tebaldi, 2004; IPCC, 2007, 2014). Assuming that changes are proceeding too fast for this key species to adapt, functional extinction is likely to occur unless cautionary measures will be taken.

Variations in morphometric traits and altered physiological performances (morphological and physiological indicators) reflect the ecological status of seagrass meadows (Ferrat et al., 2003; Romero et al., 2007). However, such descriptors yield biased-interpretations due to plant acclimation to chronic stress exposure (Macreadie et al., 2014b). Molecular level analysis could allow identifying responses to ocean changes and signatures of sub-lethal stress in marine organisms before than morphological and physiological descriptors (Evans and Hofmann, 2012). In fact, gene expression profiling, genome wide transcriptomic and proteomic analyses provide a reliable description of cell/organism responses to stress (Davey et al., 2016; Malandrakis et al., 2017). The cellular stress response (CSR) is a universal mechanism (Kultz, 2005). Whatever the organism, any cell experiencing acute environmental oscillations activates the expression of specific and conserved protein-coding genes to prevent excess damage to housekeeping components (e.g. protein denaturation and aggregation, DNA damage, cell cycle alterations, apoptosis) and eventually repairs them (Kultz, 2003, 2005). Defence mechanisms and their upstream molecular triggers can be ranked along increasing stress severity levels, providing insights into the critical threshold of environmental conditions that organisms can cope with, before mortality occurs (Evans and Hofmann, 2012). Consequently, knowledge of the expression of CSR-related genes and their associated pathways can help in predicting future consequences of global ocean change (Evans and Hofmann, 2012). Here we investigate, through a mesocosm experiment, the molecular response of *P. oceanica* when exposed to relatively short-term temperature increase. We analysed the response at different temperature levels, from natural to putatively sub-lethal for the assessed population, as the predicted levels possibly experienced by the species by the end of the century. We hypothesize different response levels will be triggered under increasing severity of thermal treatments and thus different molecular signals will be activated.

Our aim is to highlight the molecular response of *P. oceanica* when it reaches a critical temperature threshold above which it will be strongly injured by heat stress. Specific aims encompass the identification of highly responsive target genes, involved in the CSR, that can define thresholds for physiological function that underlie responses to ocean warming in *P. oceanica*. Genes are categorized in response groups, and examined through Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) gene expression analysis. Response categories are assigned colours (green, yellow and red) depending on the severity of risks associated with the activated gene-pathway-response process, figuratively visualized as a molecular traffic light. We expect our experiment to provide insights in understanding the cell stress level in seagrasses exposed to increasing water temperature as experienced in current conditions and expected under climate change scenario.

2. Materials and methods

2.1. Sampling

Posidonia oceanica sampling was carried out in spring 2016 (April 12th – 13th) in a wide and well preserved meadow located at 5 m depth off the South Eastern Spanish coastline, Isla Grosa (37° 43.7' N, 0° 42.75 W). The *in situ* monthly averaged seawater temperature ranged from a minimum of 14.26 °C ± 0.7 to a maximum of 26.26 ± 0.6 °C (Fig. A1). Average seawater temperature at the collection time was 18 °C. Since heatwaves can occur starting from late spring, we employed plants that are under a potentially risk to be exposed to anomalous heat events and presumably more sensitive to heat. In temperate seagrasses, in fact, spring plants are expected to be highly sensitive to heat, coming from winter low temperatures and having low carbohydrates content in their rhizomes. Large fragments of rooted *P. oceanica* rhizomes bearing apical growth meristems and several connected shoots were carefully collected to maintain their clonal integrity. Plants were promptly transferred into large temperature and oxygen-controlled coolers and transported to the mesocosm facility of the Oceanographic Centre in Murcia (IEO, Spain) to be transplanted within 2 h after uprooting.

2.2. Experimental set up and strategy

The mesocosm system consisted of twelve 500-L tanks (Marín-Guirao et al., 2011, 2013) where similar average number and sized *P. oceanica* fragments (i.e. ramets), bearing similar number of interlinked vertical shoots (> 30), were individually transplanted in plastic pots previously filled with coarse carbonate sediments. Four selected pots were randomly allocated inside each experimental tank (Fig. 1) and let to acclimate under the environmental conditions present *in situ* during plant sampling (temperature: 18 °C; salinity: 37.5 psμ; irradiance: 250 μmol quanta m^{-2} s^{-1}; 12 h:12 h light: dark photoperiod). During the 3 weeks of acclimation phase, temperature was increased by 0.2 °C day^{-1} up to 20 °C, which represents the *in situ* average seawater temperature during the experimental time (Fig. A1). In the meanwhile, all collected shoots were genotyped (see below) to ensure equal genotypes distribution within the experimental tanks. After the acclimation period, separate increasing thermal treatments were applied in triplicate (i.e. three experimental tanks), encompassing +20 °C (control/ambient temperature), +24 °C, +28 °C, +32 °C at a fixed heating rate of 2 °C day^{-1}. The temperature levels established as treatment conditions corresponded to the natural thermal cline recorded in the coastal area where plants were sampled, including the maximum summer critical temperature (+32 °C) predictably experienced by *P. oceanica* meadows by the end of the century in the Mediterranean Sea (Marba and Duarte, 2010; Jorda et al., 2012; IPCC, 2014). After 4 weeks of heat exposure, plant tissue was collected from four distinct ramets (all representing distinct genotypes) for each temperature treatment, one for

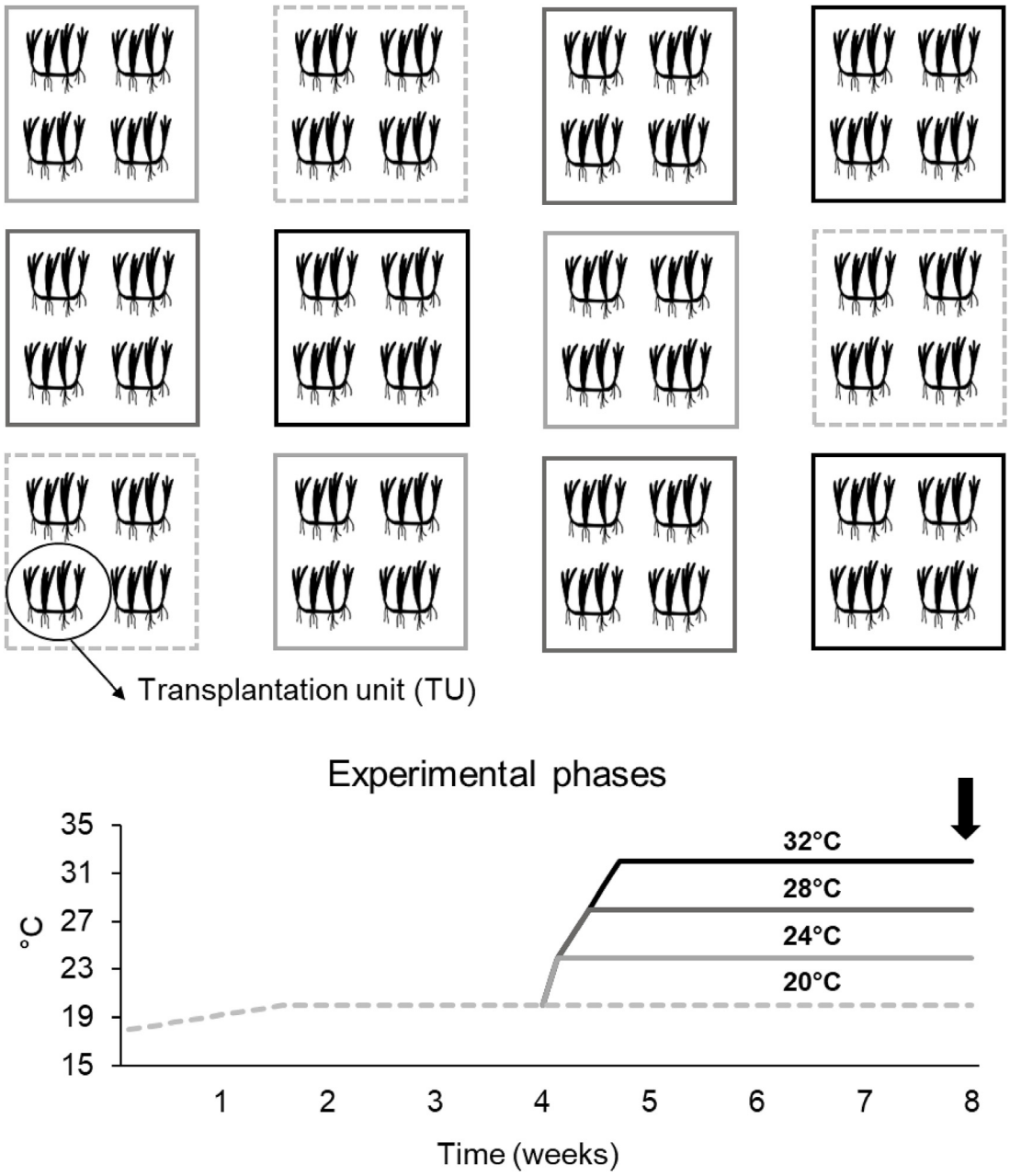

Fig. 1. Schematic representation of the experimental set up. Upper panel shows the 12 experimental tanks in which plant fragments were transplanted. Lower panel shows the different phases of the experiment and the temperatures tested. Control temperature is represented with a dashed line. The arrow shows the sampling point.

each of the three experimental tanks, plus one extra-genotype randomly chosen. A 5 cm long segment was isolated from the central portion of the first mature leaf of a shoot within each randomly selected ramet (N = 16). Epiphyte-free leaf segments for gene expression analysis were immediately preserved in RNAlater© tissue collection (Ambion, life technologies) (4 °C for 24 h) and then stored at −20 °C until RNA extraction.

2.3. Molecular analyses

2.3.1. Nucleic acids extraction

Genomic DNA extraction for genotyping was performed as in Tomasello et al. (2009) using Nucleospin Plant II kit (Macherey-Nagel) from silica-stored epiphyte-free leaf samples. Integrity and purity were checked using NanoDrop (ND-1000 UV–Vis spectrophotometer; NanoDrop Technologies) and 1% agarose gel electrophoresis. RNA from RNAlater-preserved leaf samples of the 16 selected ramets was extracted as in Mazzuca et al. (2013) with Aurum™ Total RNA Mini Kit (BIO-RAD) following manufacturer's protocol and checked for purity and integrity as for DNA. Reverse transcription of 500 ng RNA to cDNA was carried out with the iScript™ cDNA synthesis kit (BIO-RAD), according to manufacturer's instructions.

2.3.2. Genotyping

All samples were genotyped at 18 microsatellite loci (SSRs) in two separate multiplex PCR reactions consisting of 9 microsatellites each (nine-plex), in a 15 µL final volume, according to the protocol applied by Jahnke et al. (2015). Identification of distinct genotypes was assessed with the software GenClone v. 2.0 (Arnaud-Haond and Belkhir, 2007) and ramets were allocated into the tanks, accordingly. The

microsatellite analysis revealed that 44 out of 48 individuals carried distinct genotypes. However, distinct genets were allocated in each distinct experimental tank, ensuring an equal genotypic diversity among mesocosms.

2.3.3. Gene selection

Three response categories of increasing severity level were defined and associated with different colours (green, yellow and red) on the assumption that each of the three response levels involves a particular set of genes and thus induces specific cellular responses under different intensities of heat exposure, as proposed by Kultz (2003, 2005) for a general CSR mechanism. Low levels of heat stress involve protein denaturation and stimulate the synthesis of molecular chaperones, including heat-shock proteins, to re-establish native conformation of proteins. Further heating determines an upregulation of proteolysis genes, to attain protein homeostasis through the destruction of irreversibly damaged proteins. Critical heat stress induces the expression of genes that avoid the replication of damaged DNA and/or activate programmed cell death. Accordingly, we selected a list of genes of interest (GOIs) potentially involved in heat stress response on the basis of molecular functions and/or biological processes they mediate.

- The green colour was here attributed to genes encoding proteins that take an active part in protein protection, re-folding and assembly, such as the heat shock protein family and molecular chaperones. The following genes were selected: HSP90, HSP70, HSP81, sHSP, DNAJ.
- The yellow category was instead restricted to the response level ascribed to genes coding for proteins implicated in protein aggregate tagging and removal, namely ubiquitination and proteolysis, such as those participating to ubiquitin-proteasome complexes or selective protease activity. The following genes were selected: PSA3, P26S, UBC7, UBA.
- Lastly, the response level including DNA repair and apoptosis regulators enlightened the severity of ultimate cell intervention to counteract the effects of the heating. The following genes were selected and assigned to the red category: DDB, PDCD4, MCA4, DAD1, MCA1 and BI-1.

For more details about genes, protein names and specific functions see Table A.1. In agreement with previous studies (Serra et al., 2012; Marín-Guirao et al., 2016; Tutar et al., 2017), the 60 S ribosomal protein L23 (L23) and the elongation factor 1 alpha (EF1A) were taken as putative reference genes (RGs), and opportunely tested for their stability in our experimental conditions as in Serra et al. (2012). Primers for the GOIs were either available in the literature or were designed *de novo* from a *P. oceanica* transcriptome (Entrambasaguas et al., 2017) using Oligo Primer analysis v. 7.0 (Molecular Biology Insights Inc.) and Primer3 v. 0.4.0 software (Table A.1). Primer design conditions were the following: primer length 18–23 bp; amplicon length 100–250 bp; T_m~ 60 °C; GC ≥ 50%. Primer amplification efficiency was estimated from the slopes of the standard curves of the threshold cycle (CT) of five cDNA dilutions according to the equation $E = 10^{-1/slope}$. Primers' sequences, efficiencies (*E*) and regression coefficients (R^2) of RGs and GOIs are reported in Table A.1. Specificity of the amplification of newly designed primers was tested by PCR and DNA sequencing. PCR products were run on 1.5% agarose gel and intact amplicon-carrying sharp bands of expected size were excised from gel and extracted with the GenElute™ Gel Extraction Kit (SIGMA). The amplified fragments were sequenced through the Automatic Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems).

2.3.4. RT-qPCR reaction

Fast SYBR® Green Master Mix (Applied Biosystems)-based RT-qPCR reactions were performed in 384-wells plate with Viia7 Real Time PCR System (Applied Biosystems) as described in Mazzuca et al. (2013). Each reaction was conducted in triplicate with 1:10 diluted cDNA template. Three cDNA-free reactions were included as negative controls for each primer pair. Technical variation among triplicates was checked and individual values were excluded as outliers when SD > 0.3. The amplification data were analysed using the ViiA7TM Software v. 1.0 (Applied Biosystems) and the differential expression parameters were manually calculated as follows: the cycle threshold (C_T), the negative difference in cycles between the RGs and the respective GOI ($-\Delta CT = CT_{RGs} - CT_{GOI}$), the fold expression change = $\pm 2^{(|(-\Delta CT \text{ treatment})-(-\Delta CT \text{ control})|)}$.

2.4. Growth rate and necrosis

As supporting morphological evidence, plants were checked for their growth rate and necrosis throughout the experiment. Plant growth was determined according to the method proposed by Zieman (1974) by marking, in orthotropic shoots, the boundary limit between the leaf and the ligule with a needle. Three shoots of each ramet were marked right after the acclimation phase at the onset of the experimental treatment. They were subsequently collected at the end of the thermal exposure to estimate the surface area of newly formed tissue (below the mark) and thus to infer the leaf growth rate ($cm^2\ shoot^{-1}\ day^{-1}$). The surface area of leaf necrotic tissues (brown necrotic tissue on the leaf surface or at the leaf tip) was also recorded and expressed as percentage of necrotic leaf surface. Within each tank, measurements were averaged to be used as independent replicates (n = 3).

2.5. Data analysis

For molecular and phenological data, homogeneity of variance among replicates across treatments (homoscedasticity) and normal distribution of data were verified using Cochran's and Shapiro-Wilk's tests, respectively and either log- or root-transformed when needed. One-way ANOVA with four temperature levels (i.e. 20 °C, 24 °C, 28 °C and 32 °C) was conducted with STATISTICA 7 Software (StatSoft v. 7.0) to assess differences between treatments on analysed plant variables. A post hoc mean pair-wise comparison (Student-Newman-Keuls, SNK test; Zar, 1984) was performed to identify significant factor levels. To have a wide representation of how temperature affected the overall gene expression, multivariate analyses were conducted. Permutational multivariate analysis of variance, PERMANOVA (based on −ΔCT values), was performed with the PRIMER 6 & PERMANOVA + software package (Clarke and Gorley, 2006) to assess significant differences in the global expression level among experimental treatments. In case of significant results, a post-hoc pair-wise analysis was run to identify what level of the factor was responsible for the observed deviation. Results were considered significant at $p < 0.05$. Graphical exploration of the gene expression data was achieved through a multivariate Principal Component Analysis (PCA), illustrating their distribution among treatments and the putative genes underlying the stress response.

3. Results

3.1. Gene expression

Gene expression trends based on -ΔCT values for each GOI analysed, across the three response categories and at the four temperatures (20, 24, 28 and 32 °C), are illustrated in Fig. 2. ANOVA-based statistically significant differences among single GOIs expression values (-ΔCT) are reported in Table 1.

For the protein folding response category (green), significant expression changes were detected in sHSP ($p < 0.001$), that was strongly up-regulated at higher temperature, and in HSP70 ($p < 0.001$) and HSP81 ($p < 0.01$), whose expression significantly decreased as the temperature increased. No significant expression variation was observed for HSP90 and DNAJ.

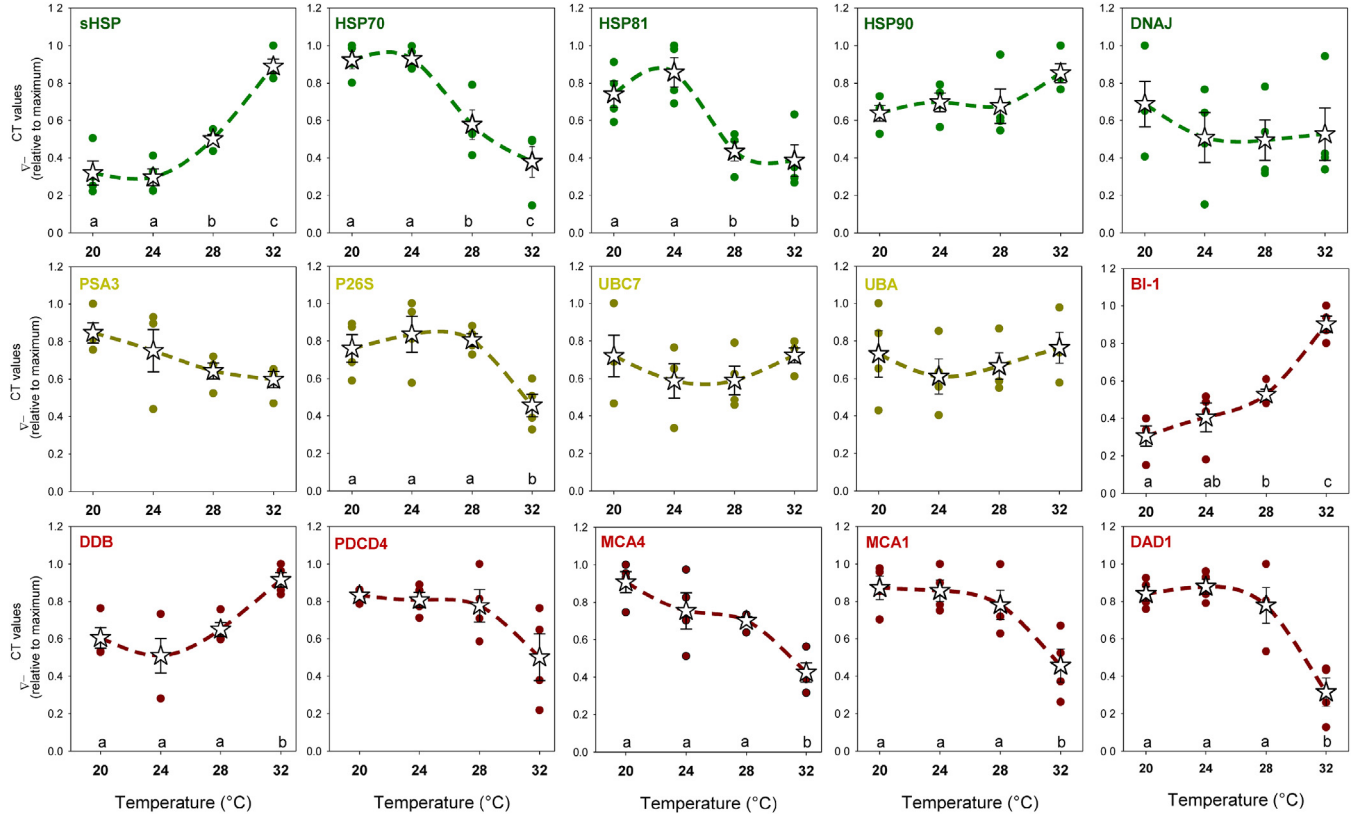

Fig. 2. Averaged single gene expression under the four thermal treatments (including the control). Gene expression refers to -ΔCT values (relative to maximum). Colours refer to the assignment criteria of response category. Letters along the x axis of each plot refer to the statistical difference estimated through pair-wise comparisons from ANOVA provided in Table 1. Stars indicate average values, SD from the mean is given as error bars. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

In the proteolysis-ubiquitination response category (yellow), P26S appeared to be significantly down-regulated at higher temperature ($p < 0.01$). PSA3, UBC7 and UBA showed no significant alterations in their expression at high temperature with respect to the control condition.

The DNA repair-apoptosis response category (red) presented significant temperature-dependent up-regulation of BI-1 ($p < 0.001$) and DDB ($p < 0.01$) and significant down-regulation of the remaining GOIs (MCA4, $p < 0.01$; MCA1, $p < 0.01$; DAD1, $p < 0.001$), with the exception of PDCD4, which showed no significant expression modification with temperature.

***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

A comparative view of differential gene expression (fold expression change) in relation to the control condition (20 °C) at the different test temperatures (24 °C, 28 °C, 32 °C) is illustrated in Fig. 3. The overall trend in fold expression change was temperature-dependent both in case of down- and upregulation. Indeed, moderate fold expression change was recorded at 24 °C for all GOIs, while only HSP70 and BI-1 showed remarkably higher expression levels at 28 °C. However, the highest fold change values resulted from plant exposure to 32 °C. Specifically, regardless of their direction, the most responsive GOIs were those belonging to the green (sHSP, HSP70) and the red (DAD1 and BI-1) response categories, reaching and even exceeding 10fold expression change compared to the control plants. With the exception of P26S, the proteolysis-ubiquitination-associated GOIs did not show neither increasing nor decreasing expression trend with temperature.

Multivariate analysis performed through the PERMANOVA test highlighted a significant effect of temperature on the global gene expression profile in *P. oceanica* ($p < 0.001$) (Table 2). Furthermore, as evidenced in the pair-wise comparisons, the gene expression at 32 °C was always significantly different from that estimated at lower temperatures ($p < 0.01$).

Graphic exploration of the overall gene expression response to temperature through a principal component analysis (PCA) is reported in Fig. 4. Gene expression data were separated according to temperature and were distributed along the principal component 1 (PC1), responsible for 71.1% of the variance. PC2 (10.2%) rather seems to explain a difference among replicates. Mean expression data at 20 °C and 24 °C partially overlapped within the 2D space, enlightening the scarce difference in the gene expression measured at these two thermal treatments. Plants exposed to 28 °C yielded mean expression data that distributed centrally and separately from the others. Finally, the 32°C-exposed plants were shifted leftward and completely separated from the rest of the thermal treatments, suggesting a stronger effect of this temperature intensity on the total gene expression, as emerged from the PERMANOVA pair-wise comparisons (Table 2). Concerning genes involved and responsible for the observed responses, strongly upregulated genes at 32 °C were projected to the left of the PC1 while the down-regulated ones were distributed to its right. In accordance with the barplot representation (Fig. 3), genes showing outstanding loading values belonged to the green and red categories of response, namely sHSP, DDB and BI-1 (< -0.3), HSP70 and DAD1 (> +0.3). The remaining GOIs were seemingly not responsible for the clustering or separation of our expression data, especially those attributed to the yellow response category, for which the expression did not vary with temperature (Table 1; Figs. 3 and 4).

3.2. Phenology

Leaf growth rate significantly decreased with thermal increase ($p < 0.05$) in a clear inverse relationship, especially marked between 20 °C/24 °C and 28 °C/32 °C. Growth rate was reduced by 81%, 57%,

Table 1
ANOVA test and pair-wise comparisons testing the effect of temperature in the response of each single GOI.

Gene Groups (response category)	Effect	df	MS	F	p	Pair-wise comparison
Protein folding (green)	HSP90					
	Temperature	3	1.051	2.331	0.126	
	Error	12	0.451			
	sHSP					
	Temperature	3	6.041	37.023	***	20 °C = 24 °C < 28 °C < 32 °C
	Error	12	0.163			
	HSP70					
	Temperature	3	13.892	18.415	***	20 °C = 24 °C > 28 °C > 32 °C
	Error	12	0.754			
	HSP81					
	Temperature	3	2.952	10.261	**	20 °C = 24 °C > 28 °C = 32 °C
	Error	12	0.288			
	DNAJ					
	Temperature	3	0.074	0.509	0.684	
	Error	12	0.144			
Ubiquination and proteolysis (yellow)	PSA3					
	Temperature	3	0.257	2.602	0.100	
	Error	12	0.099			
	P26S					
	Temperature	3	1.145	6.362	**	20 °C = 24 °C = 28 °C > 32 °C
	Error	12	0.180			
	UBC7					
	Temperature	3	0.213	0.857	0.490	
	Error	12	0.248			
	UBA					
	Temperature	3	0.113	0.514	0.680	
	Error	12	0.219			
Apoptosis and DNA repair (red)	DDB					
	Temperature	3	1.515	8.447	**	20 °C = 24 °C = 28 °C < 32 °C
	Error	12	0.179			
	MCA4					
	Temperature	3	1.616	10.207	**	20 °C = 24 °C = 28 °C > 32 °C
	Error	12	0.158			
	MCA1					
	Temperature	3	2.161	7.072	**	20 °C = 24 °C = 28 °C > 32 °C
	Error	12	0.306			
	DAD1					
	Temperature	3	16.852	15.345	***	20 °C = 24 °C = 28 °C > 32 °C
	Error	12	1.098			
	BI-1					
	Temperature	3	12.118	23.711	***	20 °C = 24 °C ≤ 28 °C < 32 °C
	Error	12	0.511			
	PCD4 (Ln-transformed)					
	Temperature	3	0.215	3.199	0.062	
	Error	12	0.067			

and 26% in plants exposed to 32 °C, 28 °C and 24 °C, respectively in comparison with control plants (Fig. 5). Plants exposed to increasing experimental temperature did not result in significant increases in leaf necrotic marks ($p > 0.05$) even if plants exposed to 32 °C showed a nearly 60% increase in necrotized tissue in respect to control conditions (Fig. 5).

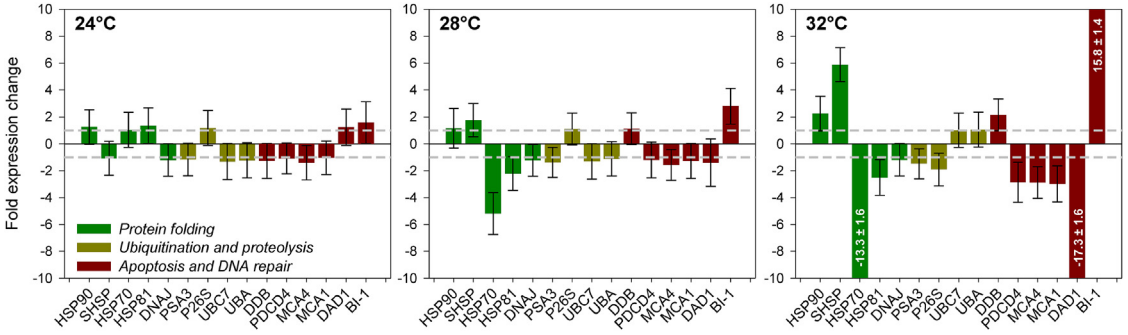

Fig. 3. Fold expression change of GOIs. Differential gene expression of GOIs under the three treatment conditions in respect to the control (20 °C). Dashed line refers to Fold Change = 1. Colours refer to the three response categories as indicated in the legend. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
PERMANOVA test and pair-wise comparisons. The effect of temperature on the overall gene expression is shown together with the pair-wise test between temperature levels. Significant differences in the paired comparisons were checked using Monte Carlo (MC) p-values due to the restricted number of possible permutations.

PERMANOVA

Source	df	SS	MS	Pseudo-F	P (perm)
Temperature	3	125.89	41.965	5.0813	***
Residual	12	99.105	8.2588		
Total	15	225			

Pair-wise Tests

Groups	**t**	**P(MC)**
20 °C, 24 °C	0.8819	0.485
20 °C, 28 °C	1.5448	0.087
20 °C, 32 °C	3.3016	**
24 °C, 28 °C	1.2823	0.195
24 °C, 32 °C	3.0535	**
28 °C, 32 °C	2.4249	**

$p < 0.001$; $^{**}p < 0.01$.

4. Discussion

4.1. Effect of temperature on the overall gene expression, leaf growth and leaf senescence

The marine environment is strongly threatened by the effects of global climatic changes. Since temperature increase is one of the main threats the marine realm is suffering, understanding the response mechanisms of species is of paramount importance. This is especially true for habitat forming species, such as seagrasses, whose meadows host a rich associated biodiversity. Here we investigated the functional limits of the highly conserved CSR under increased temperature levels (20 °C, 24 °C, 28 °C, 32 °C) in the Mediterranean seagrass *Posidonia oceanica*, providing important information for the future development of a universal molecular tool for detecting sub-lethal stress. To this aim, a molecular traffic-light was proposed as a putative response model, where the light activation pattern mimics the molecular processes involved in the CSR, and the upstream genes activated under increasing temperature intensities. We attributed the green lighting to genes involved in immediate protein folding and re-organization. Proteolysis and ubiquitination processes-related genes represented instead the yellow light. Ultimate differential expression of genes responsible for damaged DNA repair or apoptotic-related mechanisms represented, in our model, the molecular stress responses inducing the red light. Our experiment was conducted in late spring, with the rationale to utilize plants that are more sensitive to thermal stress, coming from the cold winter months.

Temperature had a strong effect on the overall gene expression in *P. oceanica*. We observed the thermal levels 20 °C and 24 °C to behave similarly as emerged from the multivariate PCA and PERMANOVA analyses (Fig. 4, Table 2). Although four degrees above the control temperature, it seems that the 24 °C treatment does not represent a real stress for *P. oceanica* since the original sampling population already experiences similar temperatures every year, as part of the natural seasonality (Fig. A1). While 28 °C and 32 °C levels induce approximately comparable expression responses, actually the former represents an intermediate but separate case. Temperatures as high as 28 °C just occurred episodically and lasted for short time in the natural population in which plants were collected (i.e. three times in the last 12 years–2006, 2015, 2017, during nine, 27 and five days respectively; Fig. A1), without leading to enhanced plant mortality (Ruiz-Fernández et al., 2016). Temperatures of 32 °C, instead, are only expected to occur by the end of the century.

Prolonged exposures to this temperature level might cause increased population decline (Olsen et al., 2012). Indeed, evidences from mesocosms experiments show that *P. oceanica* seedlings growth rate, shoot density and leaf biomass were drastically reduced upon 47–50 days of exposure to increasing temperatures ranging from 25 to 32 °C, with the lowest yield at the upper limit (Olsen et al., 2012). Seedlings mortality was also induced after one-month exposure to temperatures above 29 °C, likely due to heat-induced inhibition of photosynthesis (Guerrero-Meseguer et al., 2017). Nevertheless, a recent analysis showed that plants from our same experimental population are able to adjust their metabolism in response to several days exposure to 32 °C, through a complete transcriptomic reprogramming that allowed them to overcome and recover from the severe heat stress (Marín-Guirao et al., 2017a). It is important to stress that response to temperature vary among plants leaving in different thermal conditions, i.e. along bathymetric and latitudinal gradients. Marín-Guirao et al. (2016) reported that the detrimental effects of short-term increase in seawater temperature (32 °C), was particularly drastic on deep stands (25 m depth) compared to shallow-adapted individuals (5 m), even though plants from both depths successfully recovered after stress cessation. Further mesocosm experiments showed that populations from cold waters were more sensitive to warming than populations from warm waters, fulfilling different strategies to attempt maintaining carbon balance (Marín-Guirao et al., 2018). As ultimate escape response, cold-adapted

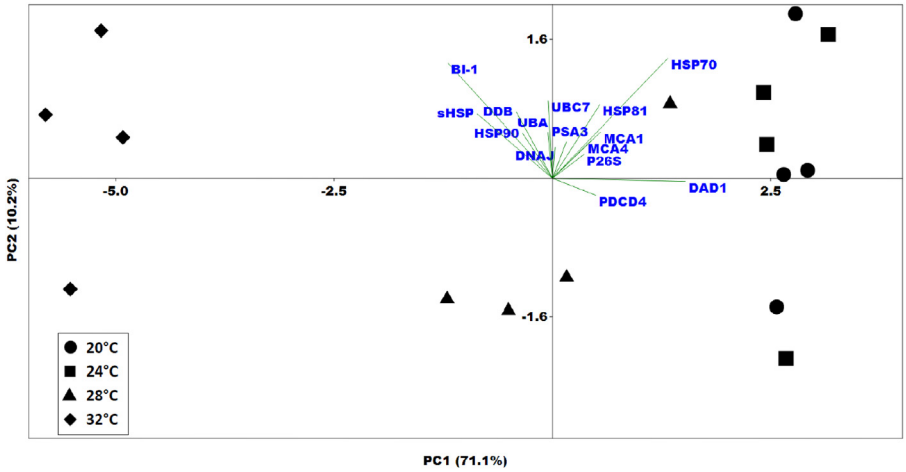

Fig. 4. Principal Component Analysis (PCA). Symbols indicate biological replicates at different experimental temperatures. Vectors indicate the relative contribution of GOIs.

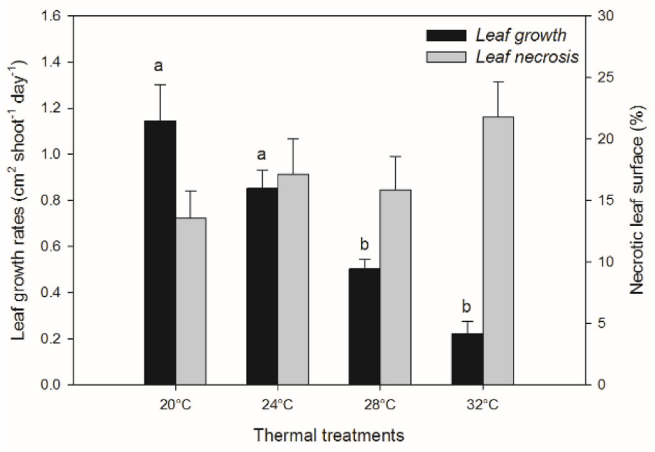

Fig. 5. Leaf growth rate and necrosis. Leaf growth rate and necrotic leaf surface were measured in control and treated plants. Letters refer to statistical difference among temperatures.

plants flowered when exposed to warming (Ruiz et al., 2018).

In our experiment, we cannot assume that tested temperatures were clearly above the species tolerance thresholds. Indeed, the progressive leaf growth reduction observed with the increase in temperature was expected, since the average optimal temperature for *P. oceanica* growth is around 18 °C, which is lower than the optimal temperature for photosynthesis (Koch et al., 2013). Terrestrial plants cope with increased temperatures diverting resources from growth to maintain plant metabolism and to activate the plant heat response (Wahid et al., 2007; Bita and Gerats, 2013). The role of temperature on seagrass growth is complex as well, due to direct and indirect interactions with other processes, such as nutrient availability and uptake, leaf senescence, resources partitioning within and among the plants and respiration (reviewed by Lee et al., 2007). In this sense, the increase in necrotized leaf tip surface of plants from the 32 °C treatment, although not significant, likely reflects the acclimative metabolic and morphological readjustment of plants through accelerated leaf senescence and resource sharing (Marín-Guirao et al., 2017b) and suggests that plants are below but close to lethal levels. Under such conditions, seagrasses as well as other terrestrial clonal plants can activate resource sharing between connected shoots within genotypes, through the so-called clonal integration (Olivé et al., 2009; Tuya et al., 2013; Dodd and Douhovnikoff et al., 2016).

Yet, despite not all the heat stress-related markers exhibited significant differential expression at 32 °C, a battery of responsive genes was detected. The observation that the expression trend we detected in our gene panel (including both responsive and not-responsive genes) was similar to what detected in different populations of *P. oceanica* previously assessed at transcriptional level under heat stress (Marín-Guirao, pers. comm.), pinpoints the importance of specific genes in conserved response mechanisms across different meadows and ecological landscapes.

4.2. The green light

Based on our gene expression data, sHSP, HSP70 and HSP81 represented the only significant green light signal recorded under high temperature exposure (Figs. 2 and 3; Table 1). Small heat shock proteins (sHSP) are known to be involved in the immediate heat stress response as ubiquitous molecular chaperones in a wide range of organisms and cellular compartments and have been found to accumulate at sub-lethal temperatures and oxidative stress in plants (Kimpel and Key, 1985; Sun et al., 2002; Wang et al., 2004). They provide an effective and low-cost thermoprotection, responsible for downstream plant thermotolerance (Wang et al., 2004). Our results concerning sHSP overexpression are in line with the data obtained in previous studies on *P. oceanica* (Marín-Guirao et al., 2016, 2017a; Tutar et al., 2017), on *Zostera marina* (Bergmann et al., 2010) and on the intertidal *Zostera noltii*, (Massa et al., 2011), both exposed to stress above their tolerance limit.

The expression of HSPs in response to warming is not always coherent among different seagrass species analysed and among different populations of the same species. In the subtidal eelgrass *Z. marina*, differences in HSPs expression were either not significant (Reusch et al., 2008) or differed among heat stressed populations, with HSP80 more responsive than HSP81 and DNAJ (Bergmann et al., 2010; Frannsen et al., 2014). Differently expressed HSPs were also recorded in *P. oceanica* from shallow and deep ecotypes (Marín-Guirao et al., 2016). A different expression pattern was found in *Nanozostera noltii* and *Cymodocea nodosa* tested under heat stress conditions similar to *Z. marina* and *P. oceanica*, respectively (Frannsen et al., 2014; Tutar et al., 2017).

In our experiment, while the sHSP was significantly up-regulated, HSP70 and HSP81 were downregulated. The difference in expression level and direction among HSPs, could relate to their functional redundancy and different sensitivity, resulting in the lower expression of the more energetically demanding ones (HSP70, 81, 90, DNAJ), and the higher expression of the more inducible sHSPs (Barua and Heckathorn, 2004; Tomanek, 2010; Haslbeck and Vierling, 2015). We can assume sHSP to represent a valid molecular marker of heat response in *P. oceanica* due to its remarkable and conserved expression behavior in contrast to the remaining heat shock proteins and molecular chaperones assessed.

4.3. The yellow light

Within the yellow response category, we assessed the trend in expression of four genes, P26S, UBC7, UBA and PA3, involved in the ubiquitin-proteasome system (UPS). The only significant signal we observed was the downregulation of P26S, while the other GOIs belonging to this category did not significantly alter their expression level (Figs. 2 and 3; Table 1). Transcriptomic analyses of *P. oceanica* tested under heat stress confirmed no differential expression of the UPS-related GOIs under study (Marín-Guirao pers. comm.).

Although ubiquitin and proteasome related genes are known to increase their expression in plants when heat stressed (Ingvardsen and Veierskov, 2001), UPS response may substantially differ in transcription levels, being the expression of related genes and the protein load variable among developmental stages and tissues (Belknap and Garbarino, 1996; Kurepa et al., 2009). It appears evident from several studies that thermotolerance acquisition in eukaryotes arises from cooperation between chaperoning and UPS mechanisms, acting in concert in a complex regulatory network, being the inhibition of UPS followed by instant upregulation of HSPs to minimize damage (Bush et al., 1997; Stone, 2014). In both animal and plants, insufficient chaperoning performance by the HSP network triggers protein tagging and breakdown processes, inducing consequent recruitment of the UPS pathway (Buckley et al., 2006; Frannsen et al., 2011).

Our hypothesis accounting for downregulation or the stable expression of the assessed GOIs belonging to the yellow category of the traffic-light model is that there is a negligible accumulation of removable proteins, due to the fast capacity of plants to attain cellular homeostasis under heat stress. This was recently described for the same population at the lipid composition level (Beca-Carretero et al., 2018). We speculate that being the green lighting chaperone system highly efficient under our experimental conditions, molecular aggregates did not accumulate in plant cells, leading to decreased activation of UPS-related genes and consequent energy saving. Contrarily to what reported in literature, P26S cannot be regarded as a powerful marker for thermal stress in *P. oceanica*, since its considerable downregulation does not result from the absence of stress but rather probably from cross-talk

with other compensating mechanisms which still confer plants alternative thermotolerance shortcuts.

4.4. The red light

The red light turned on in almost all cases at high temperature, and was characterized by strong upregulation of BI-1 and DDB, sharp downregulation of MCA1, MCA4 and DAD1 and unvaried expression of PDCD4 (Figs. 2 and 3; Table 1).

BI-1 is considered a programmed cell death (PCD) suppressor, functioning independently in mitochondria and ER related cell death pathways in plants (Xu and Reed, 1998). It is and anti-apoptotic gene, known to inhibit the BAX-mediated mitochondrial release of pro-apoptotic factors that will dictate cell death/viability induced by biotic and abiotic stresses, such as heat or pathogens, or metabolic impair (Hückelhoven, 2004; Watanabe and Lam, 2011). In heated corals, anti-apoptotic genes seem to be more responsive in the early phase of sub-lethal stress, while the pro-apoptotic ones are recruited 1 °C below the bleaching threshold, when the stress has become lethal (Ainsworth et al., 2011). This observation further supports the idea that plants in our experiment are below but near to their heat tolerance threshold. BI-1 was also extremely upregulated after only five days of exposure to 32°c, in the same population in *P. oceanica* sampled for our analysis (Marín-Guirao et al., 2017a), suggesting that plants were already suffering from thermal stress. Thus, on the basis of our observation and literature data, anti-apoptotic gene BI-1 seems to be a suitable stress marker for *P. oceanica* under heat stress.

DAD1 is another anti-apoptotic factor, known in animals and plants (Nakashima et al., 1993; Sugimoto et al., 1995; Tanaka et al., 1997), that is down-regulated in our experiment. In plants, DAD1 is a nuclear encoded gene whose product is ER localized, as a possible anchorage protein involved in calcium homeostasis and in the modulation of PCD (Danon et al., 2004). Its expression becomes almost undetectable in senescent organs, as demonstrated in *Pisum sativum* during floral senescence (Orzáez and Granell, 1997). In light of these evidences, we may further hypothesize that even though a real lethal threshold was not reached, the decay in DAD1 levels is a clear symptom of incipient PCD, another indicator of the proximity of plants to their lethal heat level.

Metacaspases represent Ca^{2+}-dependent apoptosis-promoting factors stimulating cell death, working as antagonists of BI-1 (Watanabe and Lam, 2011). Since in our analysis a downregulation of MCA1 and MCA4 was observed, we hypothesize the stress level did not reach a critical threshold for triggering the pro-apoptotic cascade in *P. oceanica*. This could also relate to the activation of sHSP, which belong to the green light category. sHSPs, in fact, are known to be implicated in PCD, interfering with apoptotic factors, preventing their release from the mitochondria and blocking caspase-dependent cell death under hyperthermia (Garrido et al., 2001; Parcellier et al., 2003; Beere, 2004).

DDB encodes a protein involved in DNA repair and genome integrity, namely nucleotide excision repair (NER) process. DNA lesions induced by several factors can affect the integrity of the double stranded DNA conformation and the processes it mediates (Manova and Gruszka, 2015). The important role exerted by DDB has been investigated in both animal and plant cells (Molinier et al., 2008; Reinardy and Bodnar, 2015). A DDB upregulation was also observed in heat-stressed *P. oceanica* shallow plants, where DDB expression decreased towards recovery (Tutar et al., 2017). DDB is thus an effective mirror of on-going NER, indicating a certain degree of DNA damage is actually occurring in *P. oceanica* at high temperature, thereby conferring this gene a powerful function of molecular stress signal.

From our overall analysis of the red light signals, BI-1, DDB and DAD1 are the genes that more strongly react to sub-lethal stress in *P. oceanica*, being highly responsive to high temperature approaching the critical upper limit of thermotolerance of the species.

5. General remarks and conclusions

Our analysis aimed to assess the functional limits of the CSR in *Posidonia oceanica* under simulated warming, looking at genes involved in the different response levels. Globally, the genes that mostly responded to high temperature belonged to the green and red categories committed to preserve housekeeping components, inhibit auto-destructive outputs and function as warning of PCD onset. The response bypasses the intermediate proteolysis-related pathway, which showed non-significant expression variation. This pattern suggests that molecular chaperoning, DNA repair and apoptosis inhibition processes are the instant light signals exerted in *P. oceanica* heat stress response. Negative modulation of metacaspases and unnecessary recruitment of UPS-related genes for poor aggregate accumulation possibly indicate efficient chaperoning effort and metabolic saving. However, although DNA repair was ensured by activation of DDB, DAD1 downregulation suggests an emerging signal of imminent cell death. It has been pointed out that apoptosis is sometimes activated as safety mechanism to preserve the organismal balance through the suppression of injured cells, but it could be that the temperature we used in the experiment was not excessively severe for *P. oceanica* plants sampled at a Mediterranean "hot" site, as result of local adaptation. Local adaptation was already shown in *P. oceanica*. Warm adapted *P. oceanica* plants showed higher resistance and tolerance to warming (Marín-Guirao et al., 2016) and plants living in different light environments, along the bathymetric gradient, saved their original response strategy when cross-transplanted in reciprocal light conditions (Dattolo et al., 2017). Indeed, *P. oceanica* undergoes strong temperature excursions in our sampling area (16–28 °C) and it may be genetically or epigenetically armored (Chinnusamy and Zhu, 2009; Mirouze and Paszkowski, 2011), minimizing the stress induced changes in gene expression, in particular at the lower temperatures used in our experiment. Nevertheless, the activated compensatory and warning mechanisms preluding imminent deadly fate for the organism, activated at 32 °C exposure, show a trend that does not reflect in the observed phenological trait variations. This is a good indication for the usefulness of molecular indicators for assessing organismal state before physiological and/or morphological damage is visible.

P. oceanica is in regression in the whole Mediterranean basin (Telesca et al., 2015) and it has been recognized as a sentinel organism due to its longevity, spread occurrence and sensitivity to anthropic disturbances (Boudouresque et al., 2000; Lopez y Royo et al., 2011). Despite the common use of morphological and physiological traits as descriptors of meadows health in *P. oceanica*, the expression of target genes has been successfully tested in the same species as predictor of population collapse in complex conditions where different stressors interact (Ceccherelli et al., 2018). Some of our proposed genes and their downstream pathways could, therefore, be targeted for further investigation and *in situ* proofing for being utilized as molecular tools when assessing *P. oceanica* performance under sub-lethal heat stress, perhaps anticipating dramatic meadow loss in the mid-term.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.marenvres.2018.07.007.

References

Ainsworth, T.D., Wasmund, K., Ukani, L., Seneca, F., Yellowlees, D., Miller, D., Leggat, W., 2011. Defining the tipping point. A complex cellular life/death balance in corals in response to stress. Sci. Rep. 1 (160). https://doi.org/10.1038/srep00160.

Arnaud-Haond, S., Belkhir, K., 2007. Genclone: a computer program to analyse genotypic data, test for clonality and describe spatial clonal organization. Mol. Ecol. Notes 7, 15–17. https://doi.org/10.1111/j.1471-8286.2006.01522.x.

Barua, D., Heckathorn, S.A., 2004. Acclimation of the temperature set-points of the heat-shock response. J. Therm. Biol. 29 (3), 185–193. https://doi.org/10.1016/j.jtherbio.2004.01.004.

Beca-Carretero, P., Guihéneuf, F., Marín-Guirao, L., Bernardeau-Esteller, J., García-Muñoz, R., Stengel, D.B., Ruiz, J.M., 2018. Effects of an experimental heat wave on fatty acid composition in two Mediterranean seagrass species. Mar. Pollut. Bull. https://doi.org/10.1016/j.marpolbul.2017.12.057.

Beere, H.M., 2004. 'The stress of dying': the role of heat shock proteins in the regulation of apoptosis. J. Cell Sci. 117, 2641–2651. https://doi.org/10.1242/jcs.01284.

Belknap, W.R., Garbarino, J.E., 1996. The role of ubiquitin in plant senescence and stress responses. Trends Plant Sci. 1 (10), 331–335. https://doi.org/10.1016/S1360-1385(96)82593-0.

Bergmann, N., Winters, G., Rauch, G., Eizaguirre, C., Gu, J., Nelle, P., Fricke, B., Reusch, T.B., 2010. Population-specificity of heat stress gene induction in northern and southern eelgrass *Zostera marina* populations under simulated global warming. Mol. Ecol. 19 (14), 2870–2883. https://doi.org/10.1111/j.1365-294X.2010.04731.x.

Bita, C.E., Gerats, T., 2013. Plant tolerance to high temperature in a changing environment: scientific fundamentals and production of heat stress-tolerant crops. Front. Plant Sci. 4 (273), 1–18 DOI: 10.3389%2Ffpls.2013.00273.

Bos, A.R., Bouma, T.J., de Kort, G.L.J., Marieke, M., van Katwijk, M.M., 2007. Ecosystem engineering by annual intertidal seagrass beds: sediment accretion and modification. Estuar. Coast Shelf Sci. 74 (1–2), 344–348. https://doi.org/10.1016/j.ecss.2007.04.006.

Boudouresque, C.F., Charbonnel, E., Meinesz, A., Pergent, G., Pergent-Martini, C., Cadiou, G., Bertrandy, M.C., Foret, P., Ragazzi, M., Rico-Raimondino, V., 2000. A monitoring network based on the seagrass *Posidonia oceanica* in the northwestern Mediterranean Sea. Biol. Mar. Med. 7 (2), 328–331.

Buckley, B.A., Gracey, A.Y., Somero, G.N., 2006. The cellular response to heat stress in the goby *Gillichthys mirabilis*: a cDNA microarray and protein-level analysis. J. Exp. Biol. 209 (14), 2660–2677. https://doi.org/10.1242/jeb.02292.

Bush, K.T., Goldberg, A.L., Nigam, S.K., 1997. Proteasome inhibition leads to a heat-shock response, induction of endoplasmic reticulum chaperones, and thermotolerance. J. Biol. Chem. 272 (14), 9086–9092. https://doi.org/10.1074/jbc.272.14.9086.

Ceccherelli, G., Oliva, S., Pinna, S., Piazzi, L., Procaccini, G., Marin-Guirao, L., Dattolo, E., Gallia, R., La Manna, G., Gennaro, P., Costa, M.M., Barrote, I., Silva, J., Bulleri, F., 2018. Seagrass collapse due to synergistic stressors is not anticipated by phenological changes. Oecologia. https://doi.org/10.1007/s00442-018-4075-9.

Chinnusamy, V., Zhu, J.K., 2009. Epigenetic regulation of stress responses in plants. Curr. Opin. Plant Biol. 12 (2), 133–139. https://doi.org/10.1016/j.pbi.2008.12.006.

Clarke, K., Gorley, R., 2006. PRIMER V6: User Manual/Tutorial. PRIMER-e, Plymouth.

Coma, R., Ribes, M., Serrano, E., Jiménez, E., Salat, J., Pascual, J., 2009. Global warming-enhanced stratification and mass mortality events in the Mediterranean. Proc. Natl. Acad. Sci. Unit. States Am. 106, 6176–6181. https://doi.org/10.1073/pnas.0805801106.

Costanza, R., d'Arge, R., de Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O'Neill, R.V., Paruelo, J., Raskin, R.G., Sutton, P., Van Den Belt, M., 1997. The Value of the world's ecosystem services and natural capital. Nature 387 (6630), 253–260. https://doi.org/10.1038/387253a0.

Danon, A., Rotari, V.I., Gordon, A., Mailhac, N., Gallois, P., 2004. Ultraviolet-C overexposure induces programmed cell death in *Arabidopsis*, which is mediated by caspase-like activities and which can be suppressed by caspase inhibitors, p35 and defender against apoptotic death. J. Biol. Chem. 279 (1), 779–787. https://doi.org/10.1074/jbc.M304468200.

Dattolo, E., Marín-Guirao, L., Ruiz, J.M., Procaccini, G., 2017. Long-term acclimation to reciprocal light conditions suggests depth-related selection in the marine foundation species *Posidonia oceanica*. Ecol. Evol. 7, 1148–1164. https://doi.org/10.1002/ece3.2731.

Davey, P.A., Pernice, M., Sablok, G., Larkum, A., Lee, H.T., Golicz, A., Edwards, D., Dolferus, R., Ralph, P., 2016. The emergence of molecular profiling and omics techniques in seagrass biology; furthering our understanding of seagrasses. Funct. Integr. GEN 16 (5), 465–480. https://doi.org/10.1007/s10142-016-0501-4.

De Boeck, H.J., Dreesen, F.E., Janssens, I.A., Nijs, I., 2011. Whole-system responses of experimental plant communities to climate extremes imposed in different seasons. New Phytol. 189, 806–817. https://doi.org/10.1111/j.1469-8137.2010.03515.x.

Díaz-Almela, E., Marbà, N., Martínez, R., Santiago, R., Duarte, C.M., 2009. Seasonal dynamics of *Posidonia oceanica* in Magalluf Bay (Mallorca, Spain): temperature effects on seagrass mortality. Limnol. Oceanogr. 54, 2170–2182. https://doi.org/10.4319/lo.2009.54.6.2170.

Dodd, R.S., Douhovnikoff, V., 2016. Adjusting to global change through clonal growth and epigenetic variation. Front. Ecol. Evol 4 (86) DOI: 10.3389/fevo.2016.00086.

Doney, S.C., Ruckelshaus, M., Duffy, J.E., Barry, J.P., Chan, F., English, C.A., Galindo, H.M., Grebmeier, J.M., Hollowed, A.B., Knowlton, N., Polovina, J., Rabalais, N.N., Sydeman, W.J., Talley, L.D., 2012. Climate change impacts on marine ecosystems. Annu. Rev. Mar. Sci. 4, 11–37. https://doi.org/10.1146/annurev-marine-041911-111611.

Duarte, B., Martins, I., Rosa, R., Matos, A.R., Roleda, M.Y., Reusch, T.B.H., Engelen, A.H., Serrão, E.A., Pearson, G.A., Marques, J.C., Caçador, I., Duarte, C.M., Jueterbock, A., 2018. Climate change impacts on seagrass meadows and macroalgal forests: an integrative perspective on acclimation and adaptation potential. Front. Mar. Sci. 5 (190), 1–23 DOI: 10.3389/fmars.2018.00190.

Duarte, C.M., 1991. Seagrass depth limits. Aquat. Bot. 40 (4), 363–377. https://doi.org/10.1016/0304-3770(91)90081-F.

Duarte, C.M., 1999. Seagrass ecology at the turn of the millennium: challenges for the new century. Aquat. Bot. 65 (1–4), 7–20. https://doi.org/10.1016/S0304-3770(99)00027-3.

Duarte, C.M., 2000. Marine biodiversity and ecosystem services: an elusive link. J. Exp. Mar. Biol. Ecol. 25, 117–131. https://doi.org/10.1016/S0022-0981(00)00194-5.

Duarte, C.M., 2002. The future of seagrass meadows. Environ. Conserv. 29 (2), 192–206. https://doi.org/10.1017/S0376892902000127.

Entrambasaguas, L., Janhke, M., Biffali, E., Borra, M., Sanges, R., Marín-Guirao, L., Procaccini, G., 2017. Tissue-specific transcriptomic profiling provides new insights into the reproductive ecology and biology of the iconic seagrass species *Posidonia oceanica*. Mar. Genom 35, 51–61. https://doi.org/10.1016/j.margen.2017.05.006.

Evans, T.G., Hofmann, G.E., 2012. Defining the limits of physiological plasticity: how gene expression can assess and predict the consequences of ocean change. Philos. Trans. R. Soc. Lond. B Biol. Sci. 367 (1596), 1733–1745. https://doi.org/10.1098/rstb.2012.0019.

Ferrat, L., Pergent-Martini, C., Roméo, M., 2003. Assessment of the use of biomarkers in aquatic plants for the evaluation of environmental quality: application to seagrasses. Aquat. Toxicol. 65 (2), 187–204. https://doi.org/10.1016/S0166-445X(03)00133-4.

Franssen, S.U., Gu, J., Bergmann, N., Winters, G., Klostermeier, U.C., Rosenstiel, P., Bornberg-Bauer, E., Reusch, T.B.H., 2011. Transcriptomic resilience to global warming in the seagrass *Zostera marina*, a marine foundation species. Proc. Natl. Acad. Sci. Unit. States Am. 108 (48), 19276–19281. https://doi.org/10.1073/pnas.1107680108.

Franssen, S.U., Gu, J., Winters, G., Huylmans, A.K., Wienpahl, I., Sparwel, M., Coye, J.A., Olsen, J.L., Reusch, T.B., Bornberg-Bauer, E., 2014. Genome-wide transcriptomic responses of the seagrasses *Zostera marina* and *Nanozostera noltii* under a simulated heatwave confirm functional types. Mar. Genom 15, 65–73. https://doi.org/10.1016/j.margen.2014.03.004.

Gambi, M.C., Nowell, A.R.M., Jumars, P.A., 1990. Flume observations on flow dynamics in *Zostera marina* (eelgrass) beds. Mar. Ecol. Prog. Ser. 61 (1–2), 159–169. https://doi.org/10.3354/meps061159.

Garrido, C., Gurbuxani, S., Ravagnan, L., Kroemer, G., 2001. Heat shock proteins: endogenous modulators of apoptotic cell death. Biochem. Bioph. Res. Co 286 (3), 433–442. https://doi.org/10.1006/bbrc.2001.5427.

Guerrero-Meseguer, L., Marín, A., Sanz-Lázaro, C., 2017. Future heat waves due to climate change threaten the survival of *Posidonia oceanica* seedlings. Environ. Pollut. 230, 40–45. https://doi.org/10.1016/j.envpol.2017.06.039.

Haslbeck, M., Vierling, E., 2015. A first line of stress defense: small heat shock proteins and their function in protein homeostasis. J. Mol. Biol. 427 (7), 1537–1548. https://doi.org/10.1016/j.jmb.2015.02.002.

Heip, C., Warwick, R., d'Ozouville, L., 1998. A European Science Plan on marine Biodiversity. European Science Foundation, Strasbourg, pp. 1–23.

Hoegh-Guldberg, O., Bruno, J.F., 2010. The impact of climate change on the World's marine ecosystems. Science 328, 1523–1528. https://doi.org/10.1126/science.1189930.

Hückelhoven, R., 2004. BAX Inhibitor-1, an ancient cell death suppressor in animals and plants with prokaryotic relatives. Apoptosis 9 (3), 299–307. https://doi.org/10.1023/B: APPT.0000025806.71000.1c.

Ingvardsen, C., Veierskov, B., 2001. Ubiquitin-and proteasome-dependent proteolysis in plants. Physiol. Plantarum 112 (4), 451–459. https://doi.org/10.1034/j.1399-3054.2001.1120401.x.

IPCC, 2007. Climate change 2007: synthesis report. In: Core writing team, Pachauri, R.K., Reisinger, A. (Eds.), Contribution of Working Group II to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. IPCC, Geneva, Switzerland 104 pp.

IPCC, 2014. Climate Change 2014: synthesis report. In: Core writing team, Pachauri, R.K., Meyer, L.A. (Eds.), Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. IPCC, Geneva, Switzerland 151 pp.

Jahnke, M., Olsen, J.L., Procaccini, G., 2015. A meta-analysis reveals a positive correlation between genetic diversity metrics and environmental status in the long-lived seagrass *Posidonia oceanica*. Mol. Ecol. 24, 2336–2348. https://doi.org/10.1111/mec.13174.

Jones, C.G., Lawton, J.H., Shachak, M., 1994. Organisms as ecosystem engineers. In: Samson, F.B., Knopf, F.L. (Eds.), Ecosystem Management. Springer, New York, pp. 130–147. https://doi.org/10.1007/978-1-4612-4018-1_14.

Jordà, G., Marbà, N., Duarte, C.M., 2012. Mediterranean seagrass vulnerable to regional climate warming. Nat. Clim. Change 2 (11), 821–824. https://doi.org/10.1038/nclimate1533.

Kimpel, J.A., Key, J.L., 1985. Heat shock in plants. Trends Biochem. Sci. 10 (9), 353–357. https://doi.org/10.1016/0968-0004(85)90111-2.

Koch, M., Bowes, G., Ross, C., Zhang, X.H., 2013. Climate change and ocean acidification effects on seagrasses and marine macroalgae. Global Change Biol. 19 (1), 103–132. https://doi.org/10.1111/j.1365-2486.2012.02791.x.

Kultz, D., 2003. Evolution of the cellular stress proteome: from monophyletic origin to

ubiquitous function. J. Exp. Biol. 206, 3119–3124. https://doi.org/10.1242/jeb.00549.
Kultz, D., 2005. Molecular and evolutionary basis of the cellular stress response. Annu. Rev. Physiol. 67, 225–257. https://doi.org/10.1146/annurev.physiol.67.040403.103635.
Kurepa, J., Wang, S., Li, Y., Zaitlin, D., Pierce, A.J., Smalle, J.A., 2009. Loss of 26S proteasome function leads to increased cell size and decreased cell number in *Arabidopsis* shoot organs. Plant Physiol. 150 (1), 178–189. https://doi.org/10.1104/pp.109.135970.
Lee, K.S., Park, S.R., Kim, Y.K., 2007. Effects of irradiance, temperature, and nutrients on growth dynamics of seagrasses: a review. J. Exp. Mar. Biol. Ecol. 350 (1), 144–175. https://doi.org/10.1016/j.jembe.2007.06.016.
Lopez y Royo, C.L., Pergent, G., Alcoverro, T., Buia, M.C., Casazza, G., Martínez-Crego, B., Pérez, M., Silvestre, F., Romero, J., 2011. The seagrass *Posidonia oceanica* as indicator of coastal water quality: experimental intercalibration of classification systems. Ecol. Indicat. 11 (2), 557–563. https://doi.org/10.1016/j.ecolind.2010.07.012.
Macreadie, P.I., Baird, M.E., Trevathan-Tackett, S.M., Larkum, A.W.D., Ralph, P.J., 2014a. Quantifying and modelling the carbon sequestration capacity of seagrass meadows – a critical assessment. Mar. Pollut. Bull. 83 (2), 430–439. https://doi.org/10.1016/j.marpolbul.2013.07.038.
Macreadie, P.I., Schliep, M.T., Rasheed, M.A., Chartrand, K.M., Ralph, P.J., 2014b. Molecular indicators of chronic seagrass stress: a new era in the management of seagrass ecosystems? Ecol. Indicat. 38, 279–281. https://doi.org/10.1016/j.ecolind.2013.11.017.
Malandrakis, E.E., Danis, T., Iona, A., Exadactylos, A., 2017. Abiotic stress of seagrasses: recent advances in transcriptomics, genomics, and systems biology. In: Kumar, M., Ralph, P. (Eds.), Systems Biology of Marine Ecosystems. Springer, Cham.
Manova, V., Gruszka, D., 2015. DNA damage and repair in plants–from models to crops. Front. Plant Sci. 6 (885), 1–26 DOI: 10.3389%2Ffpls.2015.00885.
Marbà, N., Duarte, C.M., 2010. Mediterranean warming triggers seagrass (*Posidonia oceanica*) shoot mortality. Global Change Biol. 16 (8), 2366–2375. https://doi.org/10.1111/j.1365-2486.2009.02130.x.
Marbà, N., Holmer, M., Gacia, E., Barron, C., 2006. Seagrass beds and coastal biogeochemistry. In: Larkum, A.W.D., Orth, R.J., Duarte, C.M. (Eds.), Seagrasses: Biology, Ecology and Conservation. Springer, Dordrecht, pp. 135–157. https://doi.org/10.1007/978-1-4020-2983-7_6.
Marín-Guirao, L., Bernardeau-Esteller, J., García-Muñoz, R., Ramos, A., Ontoria, Y., Romero, J., Pérez, M., Ruiz, J.M., Procaccini, G., 2018. Carbon economy of Mediterranean seagrasses in response to thermal stress. Mar. Pollut. Bull. https://doi.org/10.1016/j.marpolbul.2018.07.050 (in press).
Marín-Guirao, L., Entrambasaguas, L., Dattolo, E., Ruiz, J.M., Procaccini, G., 2017a. Molecular mechanisms behind the physiological resistance to intense transient warming in an iconic marine plant. Front. Plant Sci. 8 (1142) DOI: 10.3389/fpls.2017.01142.
Marín-Guirao, L., Ruiz, J.M., Dattolo, E., Garcia-Munoz, R., Procaccini, G., 2016. Physiological and molecular evidence of differential short-term heat tolerance in Mediterranean seagrasses. Sci. Rep. 6 (28615), 1–13. https://doi.org/10.1038/srep28615.
Marín-Guirao, L., Sandoval-Gil, J.M., Bernardeau-Esteller, J., Ruiz, J.M., Sanchez-Lizaso, J.L., 2013. Responses of the Mediterranean seagrass *Posidonia oceanica* to hypersaline stress duration and recovery. Mar. Environ. Res. 84, 60–75. https://doi.org/10.1016/j.marenvres.2012.12.001.
Marín-Guirao, L., Sandoval-Gil, J.M., García-Muñoz, R., Ruiz, J.M., 2017b. The stenohaline seagrass *Posidonia oceanica* can persist in natural environments under fluctuating hypersaline conditions. Estuar. Coast 40 (6), 1688–1704. https://doi.org/10.1007/s12237-017-0242-1.
Marín-Guirao, L., Sandoval-Gil, J.M., Ruíz, J.M., Sánchez-Lizaso, J.L., 2011. Photosynthesis, growth and survival of the Mediterranean seagrass *Posidonia oceanica* in response to simulated salinity increases in a laboratory mesocosm system. Estuar. Coast Shelf Sci. 92 (2), 286–296. https://doi.org/10.1016/j.ecss.2011.01.003.
Massa, S.I., Pearson, G.A., Aires, T., Kube, M., Olsen, J.L., Reinhardt, R., Serrão, E.A., Arnaud-Haond, S., 2011. Expressed sequence tags from heat-shocked seagrass *Zostera noltii* (Hornemann) from its southern distribution range. Mar. Genom 4 (3), 181–188. https://doi.org/10.1016/j.margen.2011.04.003.
Mateo, M.A., Romero, J., Pérez, M., Littler, M.M., Littler, D.S., 1997. Dynamics of millenary organic deposits resulting from the growth of the Mediterranean seagrass *Posidonia oceanica*. Estuar, Coast. Shelf S 44 (1), 103–110. https://doi.org/10.1006/ecss.1996.0116.
Mazzuca, S., Björk, M., Beer, S., Felisberto, P., Gobert, S., Procaccini, G., Runcie, J., Silva, J., Borges, A.V., Brunet, C., Buapet, P., Champenois, W., Costa, M.M., D'Esposito, D., Gullström, M., Lejeune, P., Lepoint, G., Olivé, I., Rasmusson, L.M., Richir, J., Ruocco, M., Serra, I.A., Spadafora, A., Santos, R., 2013. Establishing research strategies, methodologies and technologies to link genomics and proteomics to seagrass productivity, community metabolism, and ecosystem carbon fluxes. Front. Plant Sci. 4 (38), 1–19 Doi: 10.3389%2Ffpls.2013.00038.
Meehl, A., Tebaldi, C., 2004. More intense, more frequent, and longer lasting heat waves in the 21st century. Science 305 (5686), 994–997. https://doi.org/10.1126/science.1098704.
Mirouze, M., Paszkowski, J., 2011. Epigenetic contribution to stress adaptation in plants. Curr. Opin. Plant Biol. 14 (3), 267–274. https://doi.org/10.1016/j.pbi.2011.03.004.
Molinier, J., Lechner, E., Dumbliauskas, E., Genschik, P., 2008. Regulation and role of *Arabidopsis* CUL4-DDB1A-DDB2 in maintaining genome integrity upon UV stress. PLoS Genet. 4 (6), 1–12. https://doi.org/10.1371/journal.pgen.1000093. e1000093.
Moore, K.A., Shields, E.C., Parrish, D.B., 2014. Impacts of varying estuarine temperature and light conditions on *Zostera marina* (eelgrass) and its interactions with *Ruppia maritima* (widgeongrass). Estuar. Coast 37 (1), 20–30. https://doi.org/10.1007/s12237-013-9667-3.
Nakashima, T., Sekiguchi, T., Kuraoka, A., Fukushima, K., Shibata, Y., Komiyama, S., Nishimoto, T., 1993. Molecular cloning of a human cDNA encoding a novel protein, DAD1, whose defect causes apoptotic cell death in hamster BHK21 cells. Mol. Cell Biol. 13 (10), 6367–6374. https://doi.org/10.1128/MCB.13.10.6367.
Olivé, I., García-Sánchez, M.P., Brun, F.G., Vergara, J.J., Pérez-Llorens, J.L., 2009. Interactions of light and organic matter under contrasting resource simulated environments: the importance of clonal traits in the seagrass *Zostera noltii*. Hydrobiologia 629, 199–208. https://doi.org/10.1007/978-90-481-3385-7_17.
Olsen, Y.S., Sánchez-Camacho, M., Marbà, N., Duarte, C.M., 2012. Mediterranean seagrass growth and demography responses to experimental warming. Estuar. Coast 35, 1205–1213. https://doi.org/10.1007/s12237-012-9521-z.
Orth, R.J., Carruthers, T.J.B., Dennison, W.C., Duarte, C.M., Fourqurean, J.W., Heck Jr., K.L., Hughes, A.R., Kendrick, G.A., Kenworthy, W.J., Olyarnik, S., Short, F.T., Waycott, M., Williams, S.L., 2006. A global crisis for seagrass ecosystem. Bioscience 56 (12), 987–996. https://doi.org/10.1641/0006-3568(2006)56[987:AGCFSE]2.0.CO;2.
Orzáez, D., Granell, A., 1997. DNA fragmentation is regulated by ethylene during carpel senescence in *Pisum sativum*. Plant J. 11 (1), 137–144. https://doi.org/10.1046/j.1365-313X.1997.11010137.x.
Parcellier, A., Gurbuxani, S., Schmitt, E., Solary, E., Garrido, C., 2003. Heat shock proteins, cellular chaperones that modulate mitochondrial cell death pathways. Biochem. Bioph. Res. Co 304 (3), 505–512. https://doi.org/10.1016/S0006-291X(03)00623-5.
Pasqualini, V., Pergent-Martini, C., Clabaut, P., Pergent, G., 1998. Mapping of *Posidonia oceanica* using aerial photographs and side scan sonar: application off the island of Corsica (France). Estuar. Coast Shelf Sci. 47 (3), 359–367. https://doi.org/10.1006/ecss.1998.0361.
Procaccini, G., Buia, M.C., Gambi, M.C., Perez, M., Pergent, G., Pergent-Martini, C., Romero, J., 2003. The Western Mediterranean. World Atlas of Seagrasses, vol 48.
Rebetez, M., Dupont, O., Giroud, M., 2009. An analysis of the July 2006 heatwave extent in Europe compared to the record year of 2003. Theor. Appl. Climatol. 95, 1–7. https://doi.org/10.1007/s00704-007-0370-9.
Reinardy, H.C., Bodnar, A.G., 2015. Profiling DNA damage and repair capacity in sea urchin larvae and coelomocytes exposed to genotoxicants. Mutagenesis 30 (6), 829–839. https://doi.org/10.1093/mutage/gev052.
Repolho, T., Duarte, B., Dionísio, G., Paula, J.R., Lopes, A.R., Rosa, I.C., Grilo, T.F., Caçador, I., Calado, R., Rosa, R., 2017. Seagrass ecophysiological performance under ocean warming and acidification. Sci. Rep. 7 (41443), 1–12. https://doi.org/10.1038/srep41443.
Reusch, T.B.H., Veron, A.S., Preuss, C., Weiner, J., Wissler, L., Beck, A., Klages, S., Kube, M., Reinhardt, R., Bornberg-Bauer, E., 2008. Comparative analysis of expressed sequence tag (EST) libraries in the seagrass *Zostera marina* subjected to temperature stress. Mar. Biotechnol. 10 (3), 297–309. https://doi.org/10.1007/s10126-007-9065-6.
Romero, J., Martínez-Crego, B., Alcoverro, T., Pérez, M., 2007. A multivariate index based on the seagrass *Posidonia oceanica* (POMI) to assess ecological status of coastal waters under the water framework directive (WFD). Mar. Pollut. Bull. 55 (1), 196–204. https://doi.org/10.1016/j.marpolbul.2006.08.032.
Ruiz-Fernández, J.M., Bernardeau-Esteller, J., García-Muñoz, M.R., Ramos-Segura, A., 2016. Red de seguimiento de las praderas de Posidonia oceanica y cambio climático en la Región de Murcia: periodo 2004-2015. Grupo de Ecología de Angiospermas Marinas, Instituto Español de Oceanografía, Centro Oceanográfico de Murcia, Murcia 152 pp. http://www.repositorio.ieo.es/e-ieo/handle/10508/10139.
Ruiz, J.M., Marín-Guirao, L., García-Muñoz, R., Ramos-Segura, A., Bernardeau-Esteller, J., Pérez, M., Sanmartí, N., Ontoria, Y., Romero, J., Arthur, R., Alcoverro, T., Procaccini, G., 2018. Experimental evidence of warming-induced flowering in the Mediterranean seagrass *Posidonia oceanica*. Mar. Pollut. Bull. https://doi.org/10.1016/j.marpolbul.2017.10.037. (in press).
Serra, I.A., Lauritano, C., Dattolo, E., Puoti, A., Nicastro, S., Innocenti, A.M., Procaccini, G., 2012. Reference genes assessment for the seagrass *Posidonia oceanica* in different salinity, pH and light conditions. Mar. Biol. 159 (6), 1269–1282. https://doi.org/10.1007/s00227-012-1907-8.
Stone, S.L., 2014. The role of ubiquitin and the 26S proteasome in plant abiotic stress signaling. Front. Plant Sci. 5 (135). https://doi.org/10.3389/fpls.2014.00135.
Sugimoto, A., Hozak, R.R., Nakashima, T., Nishimoto, T., Rothman, J.H., 1995. dad-1, an endogenous programmed cell death suppressor in *Caenorhabditis elegans* and vertebrates. EMBO J. 14 (18), 4434–4441. https://doi.org/10.1002/j.1460-2075.1995.tb00122.x.
Sun, W., Van Montagu, M., Verbruggen, N., 2002. Small heat shock proteins and stress tolerance in plants. BBA-Gene Struct. Express 1577 (1), 1–9. https://doi.org/10.1016/S0167-4781(02)00417-7.
Tanaka, Y., Makishima, T., Sasabe, M., Ichinose, Y., Shiraishi, T., Nishimoto, T., Yamada, T., 1997. dad-1, a putative programmed cell death suppressor gene in rice. Plant Cell Physiol. 38 (3), 379–383. https://doi.org/10.1093/oxfordjournals.pcp.a029179.
Telesca, L., Belluscio, A., Criscoli, A., Ardizzone, G., Apostolaki, E.T., Fraschetti, S., Gristina, M., Knittweis, L., Martin, C.S., Pergent, G., Alagna, A., Badalamenti, F., Garofalo, G., Gerakaris, V., Louise Pace, M., Pergent-Martini, C., Salomidi, M., 2015. Seagrass meadows (*Posidonia oceanica*) distribution and trajectories of change. Sci. Rep. 5, 12505. https://doi.org/10.1038/srep12505.
Teskey, R., Wertin, T., Bauweraerts, I., Ameye, M., McGuire, M.A., Steppe, K., 2015. Responses of tree species to heat waves and extreme heat events. Plant Cell Environ. 38, 1699–1712. https://doi.org/10.1111/pce.12417.
Thomson, J.A., Burkholder, D.A., Heithaus, M.R., Fourqurean, J.W., Fraser, M.W., Statton, J., Kendrick, G.A., 2015. Extreme temperatures, foundation species, and abrupt ecosystem change: an example from an iconic seagrass ecosystem. Global

Change Biol. 21 (4), 1463–1474. https://doi.org/10.1111/gcb.12694.
Tomanek, L., 2010. Variation in the heat shock response and its implication for predicting the effect of global climate change on species' biogeographical distribution ranges and metabolic costs. J. Exp. Biol. 213 (6), 971–979. https://doi.org/10.1242/jeb.038034.
Tomasello, A., Di Maida, G., Calvo, S., Pirrotta, M., Borra, M., Procaccini, G., 2009. Seagrass meadows at the extreme of environmental tolerance: the case of *Posidonia oceanica* in a semi-enclosed coastal lagoon. Mar. Ecol. 30 (3), 288–300. https://doi.org/10.1111/j.1439-0485.2009.00285.x.
Tutar, O., Marín-Guirao, L., Ruiz, J.M., Procaccini, G., 2017. Antioxidant response to heat stress in seagrasses. A gene expression study. Mar. Environ. Res. 132, 94–102. https://doi.org/10.1016/j.marenvres.2017.10.011.
Tuya, F., Espino, F., Terrados, J., 2013. Preservation of seagrass clonal integration buffers against burial stress. J. Exp. Mar. Biol. Ecol. 439, 42–46. https://doi.org/10.1016/j.jembe.2012.10.015.
Wahid, A., Gelani, S., Ashraf, M., Foolad, M.R., 2007. Heat tolerance in plants: an overview. Environ. Exp. Bot. 61 (3), 199–223. https://doi.org/10.1016/j.envexpbot.2007.05.011.
Wang, D., Heckathorn, S.A., Mainali, K., Tripathee, R., 2016. Timing effects of heat-stress on plant ecophysiological characteristics and growth. Front. Plant Sci. 7, 1629. https://doi.org/10.3389/fpls.2016.01629.
Wang, W., Vinocur, B., Shoseyov, O., Altman, A., 2004. Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci. 9 (5), 244–252. https://doi.org/10.1016/j.tplants.2004.03.006.
Watanabe, N., Lam, E., 2011. *Arabidopsis* metacaspase 2d is a positive mediator of cell death induced during biotic and abiotic stresses. Plant J. 66 (6), 969–982. https://doi.org/10.1111/j.1365-313X.2011.04554.x.
Waycott, M., Duarte, C.M., Carruthersc, T.J.B., Orth, R.J., Dennison, W.C., Olyarnik, S., Calladine, A., Fourqurean, J.W., Heck Jr., K.L., Hughes, A.R., Kendrick, G.A., Kenworthy, W.J., Short, F.T., Williams, S.L., 2009. Accelerating loss of seagrass across the globe threatens coastal ecosystems. P. Natl. Acad. Sci. USA 106 (30), 12377–12381. https://doi.org/10.1073/pnas.0905620106.
Winters, G., Nelle, P., Fricke, B., Rauch, G., Reusch, T.B.H., 2011. Effects of a simulated heat wave on photophysiology and gene expression of high- and low-latitude populations of *Zostera marina*. Mar. Ecol. Prog. Ser. 435, 83–95. https://doi.org/10.3354/meps09213.
Xu, Q., Reed, J.C., 1998. Bax inhibitor-1, a mammalian apoptosis suppressor identified by functional screening in yeast. Mol. Cell. 1 (3), 337–346. https://doi.org/10.1016/S1097-2765(00)80034-9.
Zar, J.H., 1984. Statistical significance of mutation frequencies, and the power of statistical testing, using the poisson-distribution. Biom. J. 26 (1), 83–88. https://doi.org/10.1002/bimj.4710260116.
Zieman, J.C., 1974. Methods for the study of the growth and production of turtle grass, *Thalassia testudinum* König. Aquaculture 4 (1974), 139–143. https://doi.org/10.1016/0044-8486(74)90029-5.

Further reading

Lauritano, C., Ruocco, M., Dattolo, E., Buia, M.C., Silva, J., Santos, R., Olivé, I., Costa, M.M., Procaccini, G., 2015. Response of key stress-related genes of the seagrass *Posidonia oceanica* in the vicinity of submarine volcanic vents. Biogeosciences 12 (13), 4185–4194. https://doi.org/10.5194/bg-12-4185-2015.